Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Withdrawn claims 1-12 and 24-37 have been cancelled without prejudice, claims 13, 15, and 18 have been amended, and new claims 38-53 have been introduced.

Claims 13 and 15 have been rewritten in independent form, and claims 13, 15, 16, and 18 have been amended to specify that the recited step is "effective to enhance inhibition of the cancer cell growth, as compared to that caused by the anti-cancer agent alone" (claim 13), "effective to enhance killing of the cancer cells, as compared to the anti-cancer agent alone, and thereby treat the cancer" (claim 15), "alone, is effective to inhibit growth of the cancer cell" (claim 16), and "effective to cause cancer cell death, thereby treating the cancer" (claim 18). The comparative statements in claims 13 and 15 find descriptive support in the several examples. Claims 13 and 15 have also been modified to specify that the anti-cancer agent is a member of the recited Markush group. Descriptive support for the latter limitation is provided at pages 14-18 under the subsection heading "(2) Non-caspase inhibitor anti-cancer agents". Claim 18 has also been amended to recite that the subject is administered as "a pharmaceutical composition consisting essentially of a caspase inhibitor." No new matter is introduced by these amendments.

New claims 38-41 find descriptive support at pages 10-14 under the subsection heading "(1) Caspase inhibitors"; new claims 42 and 43 find descriptive support at pages 14-18 under the subsection heading "(2) Non-caspase inhibitor anti-cancer agents"; new claims 44-47 find descriptive support at pages 18-20 under the subsection heading "(3) Antioxidants"; and new claims 48-53 find descriptive support in the examples and accompanying figures. Therefore, no new matter has been introduced by new claims 38-53.

Claims 13-23 and 38-53 are pending, which includes independent claims 13, 15, 16, and 18. Because this application contains not more than four independent claims and thirty-six total claims (the highest number previously paid for), no excess claim fees are due with this submission. Included with this submission is a Petition for Three-month Extension of Time. All additional fees can be charged (and any overpayment credited) to Deposit Account 14-1138.

The rejection of claims 13–23 under 35 U.S.C. § 112 (1st para.) for lack of written descriptive support is respectfully traversed.

The U.S. Patent and Trademark Office ("PTO") asserts at pages 2-4 of the office action that the genus of caspase inhibitors, including the subgenera of pan caspase inhibitors, and inhibitors specific for caspase-3, caspase-8, or caspase-9, are not adequately disclosed in the specification, because the specification does not adequately disclose what agents would be encompassed by these terms and new caspase inhibitors are still being identified.

Applicants submit that the fact that members of these groups are still being identified is irrelevant; rather, the issue is whether a person of skill in the art would understand the scope of the term and whether applicants were in possession of the invention as claimed. Applicants submit that persons of skill in the art would have understood the meaning of these terms and what compounds fall within these specific subgenera (i.e., correlation of structure and function), and moreover that applicants were in possession of the invention as claimed.

The specification describes a number of caspase inhibitors on pages 10–14 of the present application, and various references cited on these pages are incorporated by reference into the body of the application. These references demonstrate that a number of peptide and peptidomimetic compounds had previously been identified in the prior art as caspase inhibitors, and that a correlation between structure and function was known. For example, U.S. Patent No. 6,197,750 to Karanewsky et al. identifies a group of C-terminal modified oxamyl dipeptides as caspase inhibitors, U.S. Patent No. 6,242,422 to Karanewsky et al. identifies a group of substituted acyl dipeptides as caspase inhibitors, U.S. Patent No. 6,187,771 to Karanewsky et al. identifies tricyclic compounds as dipeptide mimetic caspase inhibitors, U.S. Patent No. 6,184,244 to Karanewsky et al. identifies C-terminal modified (N-substituted)-2-indolyl dipeptides as caspase inhibitors, and U.S. Patent No. 6,225,288 to Han et al. identifies gamma-ketoacid dipeptides as caspase inhibitors. All of these prior U.S. patents are incorporated by reference into the present application.

Moreover, persons of skill in the art would have been fully able to identify new caspase inhibitors by screening the new compounds using known assay formats to demonstrate specificity of the new compound as an inhibitor of a particular caspase or as a pan-caspase inhibitor. One of many such assays is described, for example, in Lee et al., "Potent and Selective Nonpeptide Inhibitors of Caspases 3 and 7 Inhibit Apoptosis and Maintain Cell Functionality," *J.*

Biol. Chem. 275(21):16007-16014 (2000) (copy attached as Exhibit 1), and others are identified in the above-listed U.S. patents.

Finally, applicants demonstrate in the examples of the present application that anti-cancer agents, like BCNU or Cisplatin, when administered in combination with a variety of caspase inhibitors, including a caspase-3 inhibitor, a caspase-9 inhibitor, a caspase-8/9 inhibitor, or a pan-caspase inhibitor, can reduce the survival (i.e., enhance killing) of cancer cells such as glioblastoma cells and astrocytoma cells (*see* Figures 1-9) or colon cancer cells (*see* Figure 10) as compared to the results obtained with the anti-cancer agent alone.

Because persons of skill in the art would fully appreciate that applicants have demonstrated the improved efficacy of the combination of a caspase inhibitor and an anticancer agent (i.e., a non-caspase inhibitor) for decreasing the survival of cancer cells, and given that persons of skill of skill in the art were fully aware of the structural and functional properties of various caspase inhibitors, including how to screen new agents for specific caspase inhibitory activity, the present application satisfies the written description requirement. Therefore, the rejection of claims 13–23 under 35 U.S.C. § 112 (1^{st} para.) for lack of written descriptive support is improper and should be withdrawn.

The rejection of claims 13–23 under 35 U.S.C. § 112 (1^{st} para.) for lack of enablement is respectfully traversed.

On pages 5-9 of the outstanding office action, the PTO asserts that the present application does not enable all combinations of caspase inhibitors and non-caspase anti-cancer agents for treating all cancers, and the specification does not teach which agents would be useful to treat which cancers and at what dosage. The PTO asserts that such a disclosure would be required, because there is significant unpredictability in the activity of caspase inhibitors. Applicants disagree.

As noted above, the examples demonstrate effective killing of glioblastoma cells and astrocytoma cells using several *different* caspase inhibitors in combination with the alkylating agent BCNU and the effective killing of colon cancer cells using two *different* caspase inhibitors in combination with the platinum complex Cisplatin.

One of the striking features of cancers is that there is, indeed, a great deal of overlap in treatment approaches. For example, the nitrosureas that are used extensively in treatment of brain tumors are also used in the treatment of some lymphomas, tamoxifen (which is

primarily used to treat breast cancer) is a drug of great interest in brain tumor treatment, and 5-fluorouruacil is used in the treatment of colon cancer but is a component of cocktails used in the treatment of multiple other cancers. Moreover, the general principles that underlie the biology of cancer cells show remarkable similarities across tissue boundaries, ranging from metabolic conditions (e.g., dependence on glycolysis, tendency to be more oxidized than the primary cells from which each cancer is thought to be descended) to means of recruitment of blood vessels. In particular, cancer cells of different tissue origin share many similarities in respect to the suppression of normal cell death pathways.

Given the above-noted similarities among many cancers, persons of skill in the art would be fully able to practice the claimed invention given the positive results achieved with three different cancer types, and the combination of multiple different caspase inhibitors with two different anti-cancer agents.

For these reasons, the rejection of claims 13–23 under 35 U.S.C. § 112 (1st para.) for lack of enablement is improper and should be withdrawn.

The rejection of claims 13–23 under 35 U.S.C. § 102(b) as anticipated by WO 00/07616 to Koken et al. ("Koken"), as evidenced by corresponding U.S. Patent No. 7,217,413 to Koken, is respectfully traversed.

Koken teaches that certain combinations of caspase inhibitors with an agent that increases expression of the ProMyelocytic Leukemia ("PML") protein, such as α–, β–, or γ–interferons, can improve apoptosis of undesired cells.

With respect to claims 13-15, applicants submit that the amendments to claims 13 and 15 explicitly exclude the subject matter of Koken. In particular, claims 13 and 15 (and claimed dependent thereon) encompass the combination of a caspase inhibitor and a non-caspase inhibitor anti-cancer agent, where "the non-caspase inhibitor anti-cancer agent is selected from the group of an anti-metabolite, a DNA interactive agent, a topoisomerase inhibitor, a tubulin interactive agent, and an anti-hormonal agent." Interferons do not fall within these classes of anti-cancer agents, and given the teachings of Koken one of skill in the art would not have expected other anti-cancer agents to function in cooperation with a caspase inhibitor to induce cancer cell death. Therefore, the rejection of claims 13 and 15 (and any claims dependent thereon) is improper.

With respect to claims 16 and 18, applicants note that nowhere in Koken is it taught that caspase inhibitors themselves (or caspase inhibitors alone) may cause the death of the cells under study. Indeed, in Example 4, Koken recites as follows:

> Primary monocytes exposed to α-interferon were subjected to gradual cell death which led to the complete disappearance of the cell culture after seven days (FIGS. 3A and 3B). During the addition of zVAD with α-interferon, the death of the whole cell population was observed within 24 hours in the absence of nuclear fragmentation and of condensation of chromatin observed with interferon alone (FIGS. 3A and 3B). *Little or no cell death was observed with zVAD alone for 20 days in most of the primary cultures (8/11) (FIGS. 3A and 3B). In three cultures out of eleven, zVAD alone induced the death of part of the culture after seven days, these results probably reflecting an endogenous secretion of interferon.* Similar results were obtained with other caspase inhibitors such as DEVD."

Koken at Col. 9 (emphasis introduced). Thus, Koken suggests that only a caspase inhibitor in combination with other agents that induce PML expression, such as interferons, can be useful to induce cell death. For this reason, the limitations of claim 16 (reciting that the "introducing [of] a caspase inhibitor to the cancer cell, ...*alone*, is effective to inhibit growth of the cancer cell") and claim 18 (reciting that the subject is administered "a pharmaceutical composition *consisting essentially of* a caspase inhibitor" excludes the combination of Koken. The rejection of claims 16 and 18 (and claims dependent thereon) is therefore improper.

For these reasons, the rejection of claims 13–23 under 35 U.S.C. § 102(b) as anticipated by Koken should be withdrawn.

The rejection of claims 13–23 under 35 U.S.C. § 102(b) as anticipated by PCT Publication WO 01/27140 to Weber et al. ("Weber") is respectfully traversed.

Weber teaches the use of a number of caspase inhibitors for inhibiting non-cancer cell death during chemotherapy and radiation therapy. Although Weber specifically concerns *inhibiting non-cancer cell death*, the PTO at page 10 of the office action appears to rely on an inherency argument insofar as Weber calls for administering the caspase inhibitor in combination with a chemotherapeutic agent. Applicants submit that reliance on inherency is improper for the reasons discussed below.

Firstly, Weber does not teach or suggest each and every limitation of the claimed invention. In particular, Weber fails to teach that the combination of the caspase inhibitor and anti-cancer agent is "effective to enhance inhibition of the cancer cell growth, as compared to that caused by the anti-cancer agent alone" (as recited in claim 13) or "effective to enhance

killing of the cancer cells, as compared to the anti-cancer agent alone, and thereby treat the cancer" (claim 15). In addition, Weber most certainly fails to teach or suggest that the caspase inhibitor itself (i.e., alone) is "effective to inhibit growth of the cancer cell" (claim 16) or "effective to cause cancer cell death, thereby treating the cancer" (claim 18).

Secondly, it is a well established basis of patent law that new uses of known processes are patentable. *See* 35 U.S.C. § 101 (2008) ("Whoever invents or discovers any new and useful process … may obtain a patent therefore…."); 35 U.S.C. § 100(b) (2008) ("The term 'process' means process, art or method, and includes a new use of a known process, machine, manufacture, composition of matter, or material."). Whether or not a new use of a known process is patentable depends on whether or not the known process is "directed *to the same purpose*" as previously known processes. *See Bristol-Myers Squibb Co. v. Ben Venue Labs, Inc.*, 246 F.3d 1368, 1376, 58 USPQ2d 1508, 1514 (Fed. Cir. 2001) (emphasis added). In *Bristol-Myers*, the Federal Circuit held that claims directed to methods of treating patients for taxol-sensitive tumors by administering a certain dosage of taxol to a patient over about three hours, either with or without pretreatment of the patient for reduction of hypersensitivity to taxol, were inherently taught by a reference that reported phase I testing of taxol, using dosages and time constraints as claimed, and suggested pretreatment of patients to reduce their hypersensitivity. Importantly, the court noted that the claimed methods were *for the same purpose* as the known process described in the prior art (*id.*), and the claimed methods did not require a particular result of the recited steps (246 F.3d at 1372-73, 1378; 58 USPQ2d at 1514, 1515).

In contrast to the art and the claimed subject matter presented in *Bristol-Myers*, where the claims were held to be invalid, the purpose of the administered combination (claims 13 and 15) or administered caspase inhibitor (claims 16 and 18) is very different from that described in the cited art. In Weber, the purpose is to promote survival of non-cancer cells, whereas in the claimed invention the purpose is "*enhance* inhibition of the cancer cell growth" (claim 13, emphasis introduced), "*enhance* killing of the cancer cells…and thereby treat the cancer" (claim 15, emphasis introduced), "to inhibit growth of the cancer cell" (claim 16), and "cause cancer cell death, thereby treating the cancer" (claim 18). Because the purpose of Weber is very different from the purposes of the claimed methods, the present invention represents a patentable new use.

For all these reasons, the rejection of claims 13–23 under 35 U.S.C. § 102(b) as anticipated by Weber is improper and should be withdrawn.

Because claims 13, 15, 16, and 18 are patentable for the reasons noted above, applicants submit that new claims 38-53, which depend from one of these independent claims, are likewise patentable.

In view of all the foregoing, it is submitted that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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/Edwin V. Merkel/
Edwin V. Merkel
Registration No. 40,087

NIXON PEABODY LLP
1100 Clinton Square
Rochester, New York 14604-1792
Telephone: (585) 263-1128
Facsimile: (585) 263-1600

Exhibit 1: Lee et al., *J. Biol. Chem.* 275(21):16007-16014 (2000)

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Potent and Selective Nonpeptide Inhibitors of Caspases 3 and 7 Inhibit Apoptosis and Maintain Cell Functionality*

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Dennis Lee,[a,b] Scott A. Long,[a] Jerry L. Adams,[a] George Chan,[c] Kalindi S. Vaidya,[c] Terry A. Francis,[c] Kristine Kikly,[d] James D. Winkler,[e] Chiu-Mei Sung,[e] Christine Debouck,[f] Susan Richardson,[f] Mark A. Levy,[g] Walter E. DeWolf, Jr.,[g] Paul M. Keller,[g] Thaddeus Tomaszek,[g] Martha S. Head,[h] M. Dominic Ryan,[h] R. Curtis Haltiwanger,[h] Po-Huang Liang,[i] Cheryl A. Janson,[i] Patrick J. McDevitt,[i] Kyung Johanson,[i] Nestor O. Concha,[j] Winnie Chan,[j] Sherin S. Abdel-Meguid,[j] Alison M. Badger,[k] Michael W. Lark,[k] Daniel P. Nadeau,[k] Larry J. Suva,[k] Maxine Gowen,[k] and Mark E. Nuttall[k,l]

From the Departments of [a]Medicinal Chemistry, [c]Biomolecular Discovery, [d]Immunology, [e]Immunopharmacology, [f]Molecular Biology, [g]Molecular Recognition, [h]Physical & Structural Chemistry, [i]Protein Biochemistry, [j]Structural Biology, and [k]Bone & Cartilage Biology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

Caspases have been strongly implicated to play an essential role in apoptosis. A critical question regarding the role(s) of these proteases is whether selective inhibition of an effector caspase(s) will prevent cell death. We have identified potent and selective non-peptide inhibitors of the effector caspases 3 and 7. The inhibition of apoptosis and maintenance of cell functionality with a caspase 3/7-selective inhibitor is demonstrated for the first time, and suggests that targeting these two caspases alone is sufficient for blocking apoptosis. Furthermore, an x-ray co-crystal structure of the complex between recombinant human caspase 3 and an isatin sulfonamide inhibitor has been solved to 2.8-Å resolution. In contrast to previously reported peptide-based caspase inhibitors, the isatin sulfonamides derive their selectivity for caspases 3 and 7 by interacting primarily with the S_2 subsite, and do not bind in the caspase primary aspartic acid binding pocket (S_1). These inhibitors blocked apoptosis in murine bone marrow neutrophils and human chondrocytes. Furthermore, in camptothecin-induced chondrocyte apoptosis, cell functionality as measured by type II collagen promoter activity is maintained, an activity considered essential for cartilage homeostasis. These data suggest that inhibiting chondrocyte cell death with a caspase 3/7-selective inhibitor may provide a novel therapeutic approach for the prevention and treatment of osteoarthritis, or other disease states characterized by excessive apoptosis.

Significant advances toward understanding the molecular mechanisms of apoptosis have been achieved in recent years, and the role of caspases as integral components of the signal transduction process has been intensely studied (1, 2). Eleven human caspases have been identified thus far (3), and a critical question surrounding them is whether inhibition of downstream effector caspase(s) alone is sufficient for promoting cell survival.

Caspase-null mutants have been used to demonstrate the importance of individual family members in murine embryonic development, and cells derived from these caspase-deficient mice have allowed the association of individual caspases with specific morphological changes that occur during apoptosis (4–8). However, because these animals either die *in utero* or possess short life spans, their utility in the development of relevant disease models is severely compromised.

The involvement of caspase(s) in apoptosis is more frequently characterized *in vitro* by measuring enzyme activities and evaluating the effects of inhibitors in cell lysates and tissue extracts. Cell-based studies have been performed using peptide inhibitors with limited cell permeabilities, irreversible peptide inhibitors, and prodrug peptide inhibitors (9–11). The moderate caspase selectivities associated with these reagents have made it difficult to assess the importance of a specific caspase in apoptosis, and the interpretation of results is often ambiguous.

Caspase 3 has been found to be activated in virtually every model of apoptosis (12). It belongs to a subfamily of effector caspases, which also includes caspases 6 and 7. These caspases are activated downstream of initiator caspases such as 8 and 10. Natural substrates of caspase 3 include many proteins involved in cell maintenance and/or repair (13). For example, oligonucleosome fragmentation (DNA laddering) is a characteristic feature of apoptosis and is mediated by caspase-activated deoxyribonuclease (CAD),[1] whose activation is effected by the caspase 3-mediated cleavage of the CAD inhibitor ICAD (14). The availability of a selective inhibitor of caspase 3 would allow the evaluation of the potential for inhibition of apoptosis at the level of an effector caspase.

Osteoarthritis (OA) is a degenerative joint disease histolog-

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The atomic coordinates and structure factors (code 1QA8) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[b] To whom correspondence may be addressed. Present address: Sunesis Pharmaceuticals, 3696 Haven Ave., Suite C, Redwood City, CA 94063. Tel.: 650-556-8800; Fax: 650-556-8824; E-mail: denlee@sunesis-pharma.com.

[l] To whom correspondence may be addressed: 709 Swedeland Rd., King of Prussia, PA 19406. Tel.: 610-270-4998; Fax: 610-270-5598; E-mail: mark_e_nuttall@sbphrd.com.

[1] The abbreviations used are: CAD, caspase-activated deoxyribonuclease; OA, osteoarthritis; CHX, cycloheximide; FBS, fetal bovine serum; AMC, 7-amino-4-methylcoumarin; FMK, fluoromethylketone, *COL2A1*, type II collagen gene; TNF-α, tumor necrosis factor-α; ME, mercaptoethanol; CAM, camptothecin; CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting.

ically characterized by the erosion of articular cartilage and is a potential candidate for an anti-apoptotic therapeutic agent. Chondrocytes are the only cell type found in cartilage and are specific to this tissue. Elevated levels of apoptosis have been reported in superficial, mid-zone, and hypertrophic chondrocytes in OA cartilage (15, 16). In addition, chondrocytes adjacent to OA lesions express high levels of *bcl*-2, a gene involved in the inhibition of apoptosis (17). Cartilage degradation appears to result from cleavage of matrix proteins by proteolytic enzymes derived from chondrocytes and/or synoviocytes. As OA progresses, the fibrillar collagen network is degraded and chondrocyte cell death increases (18–21).

In several transgenic models, dysregulated apoptosis has been observed in chondrocytes associated with major structural and developmental abnormalities, thereby suggesting a critical role for the regulation of this process in articular cartilage (22–24). Increased numbers of apoptotic chondrocytes have also been observed in animal models with surgically induced OA (25). Therefore, the evidence suggesting a role for apoptosis in chondrocytes from normal cartilage is both extensive and convincing. Furthermore, during the development of OA, it has become evident that apoptotic events are dysregulated.

We have identified potent and selective inhibitors of effector caspases 3 and 7 and have evaluated their potential to inhibit apoptosis in two cell-based models. In addition, the caspase 3/7-selective inhibitors blocked apoptosis in an *in vitro* osteoarthritis model and demonstrated for the first time that caspase 3/7 activity alone is critical in chondrocyte apoptosis and that our inhibitors may lead ultimately to a novel therapeutic strategy. In addition, the compounds inhibited apoptosis in mouse bone marrow neutrophils, which have previously been shown to be dependent upon caspase 3 for apoptosis (5). Our results suggest that inhibition of caspase 3 and/or 7 is essential for the apoptosis of multiple cell types and that inhibition of this activity may represent a novel approach for the treatment of diseases characterized by excessive apoptosis, such as OA.

EXPERIMENTAL PROCEDURES

Inhibitor Synthesis—1-Methyl-5-nitroisatin is commercially available from Aldrich. Sodium 5-isatinsulfonate (Aldrich) was treated with phosphorus oxychloride in sulfalane at 80 °C to yield 5-chlorosulfonylisatin (26). Treatment of 5-chlorosulfonylisatin in tetrahydrofuran with an equivalent of amine in the presence of one equivalent of diisopropylethylamine yielded the 5-dialkylaminosulfonylisatin. 5-Dialkylaminosulfonylisatin was alkylated by treatment with sodium hydride in dimethylformamide (27) and reacting the resulting salt with an alkyl halide at temperatures ranging from 25 to 80 °C to give 1-alkyl-5-alkylaminosulfonylisatin. All compounds were characterized by satisfactory proton NMR and mass spectrometry data.

Protein Supply and Enzyme Inhibition Assays—Caspase 9 was obtained from Chemicon International, Inc. (Temecula, CA).

Expression and Purification of Caspases 3, 4, and 8—The DNA sequences encoding the caspase 3, 4, and 8 catalytic domains (no prodomain) were amplified by polymerase chain reaction from their respective full-length human caspase cDNAs using custom synthetic oligonucleotide primers and cloned into an *Escherichia coli* expression vector. The DNA sequences for an N-terminal hexa-His tag and factor Xa cleavage site were designed into the forward primers, and restriction sites were incorporated into the forward and reverse primers for cloning into the pet16b expression vector (Novagen), which was digested with restriction enzymes *Nco*I and *Xho*I (New England Biolabs). The amplified caspase DNA's cloned into pet16b were sequenced to verify correct DNA sequences. The clones were then transformed into *E. coli* expression host LW29(DE3), a derivative of BL21(DE3) containing a chromosomal LacIQ.[2] Single transformants were grown up overnight at 37 °C in LB ampicillin (100 μg/ml) in shake flasks. The overnight cultures were diluted 1/30 into fresh media and allowed to grow to an A_{600} of 0.8–1.0 before inducing with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside. The cultures were induced for 3–4 h and harvested by centrifugation. Cell pellets were frozen and stored at −70 °C.

For purification, the frozen cells, ~300 g of *E. coli* cells harvested from a 10L bioreactor, were thawed and lysed in 1.5 liters of Buffer A (25 mM HEPES, pH 8.0, 0.1% CHAPS, 10% glycerol, and 10 mM β-mercaptoethanol) containing 0.5 M NaCl. After centrifugation, the supernatant was applied to a nickel-nitrilotriacetic acid column, which had previously been equilibrated with Buffer A. N-terminal His-tagged caspases were eluted with 300 mM imidazole in Buffer A. The eluate was dialyzed against Buffer A to remove the imidazole and salt and applied to a ToyoPearl DEAE-650M column. Caspases were eluted with a linear salt gradient, 0–500 mM NaCl, in Buffer A. A typical yield was ~300 mg of each caspase. Purified proteins were characterized by N-terminal sequencing and matrix-assisted laser desorption/ionization mass spectrometry, which confirmed that each caspase was composed of equal molar of p20 and p10 subunits, and molecular weights were as expected from the cDNA sequence.

Expression and Purification of Caspases 1, 2, 6, and 7—For caspases 2, 6, and 7, the full-length DNA sequences containing an N-terminal hexa-His tag were cloned and expressed in *E. coli* as described above. After lysis of the cells, the presence of proteolytically processed enzyme was detected in the supernatant by Western blot with polyclonal antibody directed against the N-terminal peptide of each p20 subunit. The supernatant was applied to a ToyoPearl DEAE 650 M column and the active catalytic domain (p10/p20 complex) was eluted with a salt gradient as described above. Partially purified p10/p20 complex of each caspase was used for compound screening assays without further purification. For caspase 1, the N-terminal hexa-His-tagged proenzyme, which was expressed as an insoluble pellet, was solubilized in 8 M urea in buffer B (50 mM $NaPO_4$, pH 7.5, 0.5 M NaCl) and applied to an nickel-nitrilotriacetic acid column. Procaspase 1 was refolded in the nickel-nitrilotriacetic acid column by washing the column with Buffer B and eluted with 300 mM imidazole in Buffer B. The proenzyme was desalted using a Sephadex G-25 column equilibrated with Buffer A and adjusted to 5 mM dithiothreitol. Activation of proenzyme to p10/p20 complex was achieved by concentrating to 4 mg/ml and incubating 2 days at 4 °C. The activated protein mixture was further fractionated using Superdex 200. Fractions containing caspase 1 activity were pooled. SDS-polyacrylamide gel electrophoresis showed that the active enzyme was >90% pure.

Enzyme Inhibition Assays—Enzyme assays were run in 200-μl volumes and contained the following: 25 mM K^+HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 5 mM β-mercaptoethanol (β-ME), and 10 μM Ac-YVAD-AMC (caspase 1); 50 mM sodium acetate, pH 6.2, 10% glycerol, 0.25 mM EDTA, 5 mM β-ME, and 25 μM Ac-VDVAD-AMC (caspase 2); 25 mM K^+HEPES, pH 7.5, 0.1% CHAPS, 50 mM KCl, 5 mM β-ME, and 10 μM Ac-DEVD-AMC (caspases 3 and 7); 25 mM KOAc, pH 5.8, 1 mM EDTA, 10% sucrose, 0.1% CHAPS, 5 mM β-ME, and 100 μM Ac-LEED-AMC (caspase 4); 50 mM Tris-HCl, pH 7.4, 0.1% CHAPS, 1.5 mM $MgCl_2$, 1 mM EDTA, 5 mM β-ME, and 10 μM Ac-DEVD-AMC (caspase 6); Na^+MOPS, pH 7.5, 10% glycerol, 0.25 mM EDTA, 5 mM β-ME, and 10 μM Ac-IETD-AMC (caspase 8); or 100 mM Na^+MES, pH 6.5–10% polyethylene glycol 8000, 0.1% CHAPS, 10 mM β-ME, and 10 μM Ac-LEHD-AFC (caspase 9). Recombinant caspases were diluted into the appropriate buffer to about 10 units/assay (1 units = 1 pmol of AMC product formed/min) and were added to the above incubation mixtures. All inhibitors tested were diluted into Me_2SO prior to addition to the assay mixture; the final Me_2SO concentration was 5%. Accumulation of AMC was measured at 30 °C with a Cytofluor 4000 fluorescent plate reader (Perseptive Biosystems) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Crystallography—Co-crystals of the complex between recombinant human caspase 3 and inhibitor 4 were grown from 4-μl hanging drops formed by mixing equal volumes of protein (10 mg/ml in 20 mM HEPES, pH 7.0) and reservoir solution. The drops equilibrated at room temperature (21 °C) through the vapor phase against 500 μl of a reservoir solution that contained 15–18% polyethylene glycol 6000, 0.1 M sodium citrate, pH 5.9, 20 mM L-cysteine, and 5% glycerol. The crystals belong to the space group I222 with unit cell dimensions, a = 67.2 Å, b = 83.3 Å, c = 96.0 Å. Diffraction data were collected at the X12B beamline of the National Synchrotron Light Source (Brookhaven National Laboratory, Upton, NY) from a single flash-frozen crystal with its largest dimension of less than ≈20 μm. The structure of the caspase 3 - inhibitor 4 complex was determined by difference Fourier using the coordinates of the isomorphous caspase 3 complex with Ac-DEVD-dimethylbenzoate previously determined and refined to 2.0 Å (data not shown). Initially, the difference electron density maps showed a 3σ peak in the active site. The interpretation of the map was aided by

[2] SmithKline Beecham, unpublished data.

assuming a proposed mode of inhibition in which the thiolate group of Cys[163] is covalently linked to C-3 of the inhibitor and the crystal structure of epi-**5**. The model was refined in X-PLOR (28, 29), and O (30) was used for manipulation of the model. The average *B*-factor for protein atoms is 16.2 Å^2 and for non-protein atoms is 30.0 Å^2. The model of the asymmetric unit includes residues 29–173 and 185–277 of caspase 3, 45 water molecules, and the inhibitor molecule **4**.

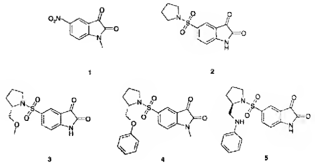
Neutrophil Apoptosis Assay—Mouse bone marrow cells were treated with 10 μg/ml cycloheximide (CHX) at 2×10^6 cells/ml in RPMI medium (Life Technologies, Inc.) containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT), penicillin (10 units/ml), and streptomycin (50 ng/ml) (Life Technologies, Inc.) in 5-ml round-bottomed polypropylene tubes for 3 h at 37 °C; inhibitors were added at the same time as CHX. Approximately half of the cells were used for evaluation of neutrophil viability by FACS analysis, and the remainder was used for a DNA ladder assay as described previously (8). To determine neutrophil viability following CHX treatment, cells were washed and stained with anti-Gr-1-fluorescein isothiocyanate (PharMingen, San Diego, CA), and propidium iodide and analyzed by flow cytometry. This procedure was used also to assess propidium iodide staining of chondrocytes. Samples were analyzed by FACS. For the DNA ladder assay, DNA released into the cytoplasm was extracted, run on a 1% agarose gel, and stained with ethidium bromide (8).

Chondrocyte Cell Culture—The immortalized human chondrocyte cell line C20.A4 (31) was grown in 50/50 DMEM/Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, Inc.), penicillin (10 units/ml), and streptomycin (50 ng/ml) (Life Technologies, Inc.).

Bovine chondrocytes were isolated from the articular cartilage of histologically normal carpal metacarpal joints of calves (0–3 months of age). Chondrocytes were isolated by sequential enzyme digestion as follows. Cartilage (2 joints) was incubated in 50 ml of 0.2% testicular hyaluronidase (type I-S from bovine testes) in DMEM without serum for 20 min at 37 °C. The hyaluronidase was then removed, and the tissue was incubated in 50 ml of 0.25% Pronase E in DMEM without serum for 20 min at 37 °C. The Pronase E was removed, and the cartilage was then incubated with 0.2% *Clostridium histolyticum* collagenase D (Roche Molecular Biochemicals) in DMEM plus 10% FBS overnight at 37 °C. The digested tissue was filtered through a 100-μm cell filter (Falcon), and the cells were pelleted by centrifugation (1200 × *g* for 10 min). The primary bovine chondrocytes were grown routinely in Ham's F-12 medium supplemented as above plus antibiotic-antimycotic (Life Technologies, Inc.). All cells were cultured at 37 °C in a humidified atmosphere of 95% air, 5% CO_2. Upon reaching confluence, the cells were subcultured using trypsin-EDTA (Life Technologies, Inc.).

Chondrocyte Cell Death ELISA—Human chondrocyte cells (C20/A4) (31) or primary bovine chondrocytes were grown in 24-well plates at 20,000 cells/well overnight and then treated with either camptothecin alone (4 μg/ml) (Biomol) or camptothecin (4 μg/ml) and Z-VAD-FMK (50 μM) or isatin sulfonamides for 24 h. Cell lysates were prepared by combining the cells from the monolayer with the cells, which detached during the treatment period. Cells floating in the media were pelleted by centrifugation for 5 min at 1,000 × *g*, resuspended in the manufacturer's lysis buffer, and then added back to the monolayer, and the total cell population was brought to 500 μl with lysis buffer. The samples were lysed for 30 min at 4 °C and then centrifuged for 10 min at 14,000 × *g* to clarify the lysates. Samples were evaluated for mono- and oligonucleosome DNA fragment formation in the cell death ELISA following the manufacturer's protocol (Roche Molecular Biochemicals).

Chondrocyte Promoter Reporter Assay—Human chondrocyte cells (C20/A4) (31) were stably transfected, and primary bovine chondrocytes were transiently transfected with a construct (pGL3-Basic-NEO^r-COL2A1) in which the regulatory sequence (−577 to +3426) (32) of the type II collagen (*COL2A1*) gene is driving a luciferase reporter gene. To prepare the expression vector, the neomycin-resistant gene (NEO^r) was inserted at the *Bam*HI site of the pGL3-Basic vector (Promega). The 4.0-kb *COL2A1* fragment was inserted at the *XhoI/MluI* sites and transfected into the C20/A4 cells and primary bovine chondrocytes using the calcium phosphate transfection method (Invitrogen). For stable cell line generation, cells were grown overnight in 10-cm dishes at 1×10^6 cells/dish and transfected with 20 μg of pGL3-Basic-NEO^r-COL2A1. Single clones were selected by growth in Geneticin (G418) (Life Technologies, Inc.) at 400 μg/ml for 2–3 weeks. Positive cells grown from single clones were maintained in media containing 200 μg/ml G418. Transiently transfected primary bovine cells and stably transfected C20A/4 cells were seeded in 24-well plates at 25,000 cells/well overnight and then treated with either camptothecin alone (4 μg/ml) or camptothecin (4 μg/ml) with Z-VAD-FMK (50 μM) or isatin sulfonamides for 8 h. Cells were then washed with phosphate-buffered saline and lysed with 100 μl/well of the manufacturer's lysis buffer (Promega) for 15 min while rocking at room temperature. Lysates were centrifuged for 30 s at 14,000 × *g*, and the clear lysate was transferred to a new tube prior to reporter analysis. Samples (20 μl) were transferred to a 96-well luminescence detection plate and reacted with 100 μl of the luciferase assay reagent (Promega), which was injected by a Microlumat LB96P luminometer (Wallac) in order to measure luciferase activity.



1 2 3 4 5

FIG. 1. **Caspase inhibitors.**

Statistical Analysis—For each parameter, differences between groups were assessed by unpaired Student's *t* test using Excel (Microsoft Corp.). Differences with a value of $p < 0.05$ were considered significant.

RESULTS

High throughput screening for inhibitors of recombinant human caspase 3 resulted in the identification of 5-nitroisatin (**1**, Fig. 1), which inhibited the enzyme with a $K_{i(app)}$ of 0.5 μM (Table I). The potency of this compound and the non-peptidic nature of its structure made it an attractive starting point for a drug discovery effort.

Using the previously determined x-ray structure of caspase 3 (33), an initial binding hypothesis was developed. Our model was based on the assumption that a tetrahedral intermediate between the catalytic cysteine residue and the ketone carbonyl group of inhibitor **1** is formed (34). The mode of binding suggested that extension of the molecule from the 5-position would allow access to the S_2-S_4 (35) regions of the active site. In addition, there are metabolic stability issues associated with nitroaromatics. Therefore, a chemical functionality that would allow for the facile incorporation of chemical diversity was sought as a replacement for the 5-nitro group (36). Evaluation of a series of groups resulted in the identification of isatin sulfonamide **2**, which retained significant activity against caspase 3 ($K_{i(app)}$ = 1.4 μM). Relative to lead **1**, isatin sulfonamide **2** possessed much improved selectivity for caspases 1, 3, and 7 (Table I). Preparation of an extensive series of compounds with variation of groups about the sulfonamide functionality and isatin nitrogen culminated in the identification of isatin sulfonamides **3** and **4**, 60 and 15 nM inhibitors of caspase 3, respectively. In addition, these inhibitors exhibited 100-fold or greater selectivity for the highly homologous caspases 3 and 7 (13) *versus* all other family members except caspase 9. Against this caspase, the selectivity ranged from 10- to 50-fold.

The mechanism of inhibition *versus* caspase 3 was demonstrated to be reversible and competitive with respect to the substrate Ac-DEVD-AMC. In addition, no evidence for time dependence was observed. Reversibility was assessed by preincubating recombinant human caspase 3 with inhibitor **3**. Samples of this mixture at several time points up to 2 h were diluted 40-fold into assay buffer, and enzyme activity was measured with the substrate Ac-DEVD-AMC. The observed inhibition at all time points did not vary significantly over time

TABLE I
Inhibitor selectivity of the isatin sulfonamides
Inhibitor studies were performed as described under "Experimental Procedures."

Inhibitor	$K_{i(app)}$ [a]							
	Caspase 1	Caspase 2	Caspase 3	Caspase 4	Caspase 6	Caspase 7	Caspase 8	Caspase 9
				μM				
1	12	>50	0.50	4.0	1.7	0.29	>50	21
2	9.0	>50	1.4	>50	>50	3.0	>50	22
3	>25	>25	0.060	>25	>25	0.17	>25	3.1
4	17	4.9	0.015	33	29	0.047	49	0.46
5	>50	11	12	>50	57	10	>50	>50

[a] The $K_{i(app)}$ (μM was calculated from the estimated IC_{50} using the following equation assuming competitive inhibition, which is characteristic for this class of compounds, $K_{i(app)} = IC_{50}/(1 + [S]/K_S)$, where $[S]$ is the concentration of the substrate in the assay and K_S is the Michaelis constant of the substrate.

and was consistent with that expected for a freely and rapidly reversible inhibitor. Isatin sulfonamide **3** was competitive *versus* caspase 3 with a K_i of 83 nM (data not shown).

The basis of this unique selectivity for caspases 3 and 7 was evaluated by examining the protein sequence alignments of the caspases against which the compounds were screened. Since these inhibitors were initially modeled to interact with the catalytic cysteine residue, we focused on those protein residues proximal to this region of the active site. This analysis revealed that the observed selectivity was likely due to three hydrophobic residues in the S_2 pocket (Tyr^{204}, Trp^{206}, and Phe^{256}) that are unique to caspases 3 and 7 (Table II). In addition, the sequence alignments of caspases 5, 10, and 13, which were not available for testing directly, suggest that the isatin sulfonamides may not inhibit these family members as potently as caspases 3 and 7. The residues corresponding to Tyr^{204} and Trp^{206} of caspase 3 are Val/Trp (caspases 5 and 13) and Tyr/Phe (caspase 10). The residue corresponding to Phe^{256} of caspase 3 for each of these caspases cannot be determined because of variability in loop size in this portion of the protein.

A 2.8-Å resolution x-ray co-crystal structure of the complex between recombinant human caspase 3 and isatin sulfonamide **4** was obtained. Data collection and statistics are given in Table III. The electron density map showed good supporting density for all atoms of the isatin framework and pyrrolidine ring but weak density for the phenoxymethyl side-chain (Fig. 2*A*). Compared with the previously determined structure of caspase 3 (33), there are no significant differences in conformation of the amino acid side chains of the protein.

As predicted by our initial hypothesis, the x-ray co-crystal structure reveals that a tetrahedral intermediate is formed between the catalytic cysteine thiolate and the isatin ketone carbonyl group (Fig. 2*B*). The S_2 pocket is involved in extensive hydrophobic contacts with the pyrrolidine ring of the inhibitor and supports our initial proposal of the importance of this hydrophobic pocket for conferring specificity to the isatin sulfonamides. As predicted, inhibitor **1**, which does not possess the pyrrolidine ring, exhibits only moderate selectivity between the caspases.

The S_1 subsite of the caspases confers high selectivity for the cleavage of substrates possessing a P_1 aspartic acid (37–39). In the x-ray co-crystal structure, this subsite is occupied only by a water molecule. Despite the absence of inhibitor binding in the S_1 subsite, a representative set of isatin sulfonamides (inhibitor **3** and two closely related analogs) exhibited little inhibition of other cysteine proteases such as cathepsins B, L, K, and S (IC_{50} values $\gg 5$ μM; data not shown) (40). In addition, these compounds exhibited little inhibition of human recombinant calpain I, a protease implicated in apoptosis (41) (<20% inhibition at 50 μM) (data not shown).

During peptide substrate hydrolysis, the "oxyanion pocket" formed by the backbone amide NHs of Cys^{163} and Gly^{122} is

TABLE II
Structural basis for caspase selectivity
Sequence alignment about S_2 subsite is shown. Residue numbers correspond to those of caspase 3. "?" indicates that the identity of the amino acid at this position is ambiguous from the alignment, and "—" indicates that there is no corresponding residue.

Caspase	Residue		
	204	206	256
1	V	W	—
2	A	M	F
3	**Y**	**W**	**F**
4	V	W	?
6	Y	H	A
7	**Y**	**W**	**F**
8	V	Y	?
9	V	W	?

TABLE III
X-ray data collection and refinement

Data collection statistics	
Resolution (Å)	50–2.8
No. of observations	113,422
Completeness (%)	96.6 (82.4)[a]
Redundancy	4.5
R_{sym}	0.174 (0.267)[a]
$I/\sigma(I)$	4.3
Refinement statistics	
Resolution (A)	40.0–2.8
No. of reflections, $F > 2\sigma(F)$	6,386
R	0.214
R_{free}[b]	0.297
Non-H atoms[c]	1998
Root-mean-square deviation from standard geometry[d]	
Bond length	0.01 Å
Bond angles	1.3 °

[a] Highest resolution shell.
[b] Using 10% of reflections.
[c] Protein-bonded atoms.
[d] Deviations from the standard bond lengths and bond angles compiled by Engh and Huber (49).

involved in stabilization of the negatively charged oxygen atom of the tetrahedral intermediate. In the caspase 3/inhibitor **4** structure, the oxygen of the tetrahedral intermediate and the amide carbonyl oxygen are within hydrogen bonding distance of Cys^{163}NH and Gly^{122}NH, respectively. There is a hydrophobic and/or aromatic interaction between the edge of Tyr^{204} with one face of the bicyclic isatin core, which likely contributes to the binding of the inhibitor. The phenoxymethyl side chain fits the data best when positioned in the shallow S_3 pocket lined by residues Ser^{65}, Arg^{207}, and Ser^{209}. However, this density is weak (Fig. 2*A*), and a subsequent co-crystal structure with a closely related analog of inhibitor **4** shows strong electron den-

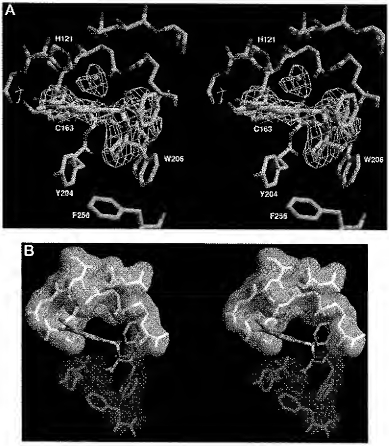


FIG. 2. *A*, $2F_o - F_c$ electron density map. Stereo view of the final $2F_o - F_c$ electron density map around the inhibitor and water molecule contoured at 1 σ level. The map was computed using data between 6.0 and 2.8 Å and model phases. A covalent bond links the inhibitor C-3 atom to $Cys^{163}S\gamma$, the pyrrolidine ring binds in the S_2 binding pocket, and the phenoxy ring occupies the shallow S_3 binding site. In this complex the S_1 pocket is occupied by Wat^{518}. The figure was prepared with BOBSCRIPT (47) and Raster3D. *B*, crystal structure of enzyme/inhibitor 4 complex. Stereo view of the molecular surface representation of the caspase 3 active site in complex with isatin sulfonamide 4. The catalytic residues His^{121} and Cys^{163} are colored by atom, the S_1 pocket is the unoccupied region behind the isatin ring (shown in *black*), and the hydrophobic S_2 pocket formed by Tyr^{204}-Phe^{256}-Trp^{206} (left to right) is shown in *magenta*. Carbon atoms of inhibitor 4 are *gray*; oxygen atoms are *red*, nitrogen atom is *blue*, and sulfur atom is *yellow*. The figure was generated using the program MOLMOL (48).

sity for this side chain on the surface of Phe^{256}.[3] In addition, in the caspase 3/inhibitor 4 structure, Ser^{58} of a crystallographic symmetry-related molecule occludes this region about Phe^{256}, and likely influences the observed positioning of the inhibitor side chain.

We next evaluated the ability of isatin sulfonamide 4 to functionally inhibit apoptosis in a caspase 3-dependent cell-based model of apoptosis. Apoptosis as measured by dye exclusion in mouse bone marrow neutrophils derived from caspase $3^{ex3-/-}$ mutants had previously been shown to be dependent upon caspase 3 (5). We therefore treated wild-type neutrophils with cycloheximide to accelerate apoptosis, and cell viability in the presence and absence of inhibitor was measured after 3 h. A dose-dependent decrease in apoptosis as measured by double labeling for Gr-1 and propidium iodide exclusion following treatment with inhibitor 4 was observed. At 25 μM, there was an 80% reduction in cell death (Fig. 3*A*). In addition, there was a complete abrogation of DNA laddering at 25 μM (Fig. 3*B*). Since apoptosis is dependent upon caspase 3 in these cells (5), the difference between *in vitro* enzyme (IC_{50} = 30 nM) and cellular apoptosis ($IC_{50} \approx$ 10 μM) inhibition potencies is suggestive of protein binding and/or limited cell penetration of the inhibitor.

To support this proposal, we demonstrated that the *in vitro* inhibition of recombinant human caspase 3 by isatin sulfonamide 4 in 30% Jurkat cell cytosol/buffer resulted in a 50-fold attenuation of inhibitor potency (data not shown). Full inhibitor activity was recovered upon dilution of the inhibitor/cytosol mixture with buffer (inhibitor potency is similar to that observed in 100% buffer), indicating that it is a reversible binding event between inhibitor and cytosolic constituents. The nature and/or target(s) of this interaction is unknown at this time. Despite the attenuation in inhibitor potency observed in neutrophils, these results clearly establish the utility of inhibitor 4 as a tool to study the importance of caspases 3 and 7 in cellular apoptosis.

The ability of these compounds to inhibit apoptosis was next examined in chondrocytes. Due to the scarcity of primary human chondrocytes, we used primary bovine chondrocytes and an immortalized human chondrocyte cell line C20/A4 (32) to investigate the effect of inhibitor 4 and a related compound with much lower activity, isatin sulfonamide **5**, on chondrocyte apoptosis. The residues of caspase 3 with which the inhibitor interacts are identical between the bovine and human enzymes. Camptothecin-induced apoptosis, determined by cell death ELISA, was blocked completely by treatment with the non-selective, irreversible peptide inhibitor Z-VAD-FMK (10) at 50 μM (Fig. 4, *A* and *B*). Treatment of C20/A4 cells and primary bovine chondrocytes with the caspase 3/7-selective inhibitor 4 also blocked cell death (IC_{50} = 6 μM). Compound 4 was nontoxic at all concentrations tested as measured by trypan blue exclusion (results not shown). In addition, propidium iodide staining was also inhibited by isatin sulfonamide **4** (Fig. 5). The structurally related analog **5**, which is a weak inhibitor of caspase 3 (Table I), failed to block cell death at concentrations up to 25 μM (Fig. 4, *A* and *B*).

To examine the consequence(s) of inhibiting chondrocyte apoptosis, the activity of the transfected type II collagen promoter-luciferase reporter was investigated in stably transfected C20/A4 cells and transiently transfected primary bovine chon-

[3] D. Lee, S. A. Long, J. L. Adams, K. Kikly, J. D. Winkler, C.-M. Sung, M. A. Levy, D. P. Nadeau, M. E. Nuttall, and W. E. DeWolf, Jr., manuscript in preparation.

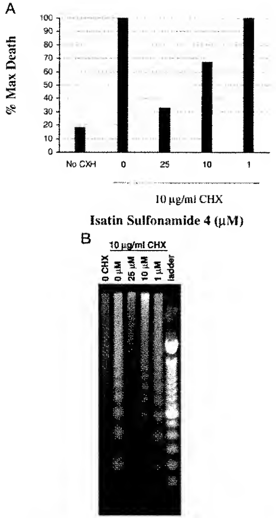


FIG. 3. **Inhibition of neutrophil apoptosis.** Mouse bone marrow neutrophils were treated with or without 10 μg/ml CHX for 3 h at 37 °C in the absence or presence of the caspase 3/7-selective inhibitors. Apoptosis was analyzed by FACS (*A*) and DNA laddering (*B*). *A*, treated cells were washed and stained with anti-Gr-1-fluorescein isothiocyanate and propidium iodide was added to asses viability. Compound **4** dose-dependently inhibited propidium iodide staining of neutrophils treated with CHX (*A*). *B*, DNA laddering analysis: the *first lane* is a control with no CHX and treatment with varying concentrations of inhibitor **4** in the presence of CHX are in the following *four lanes*. The *final lane* is a standard of 100-base pair DNA fragments. These experiments were run in triplicata, and similar inhibitor dose responses were observed.

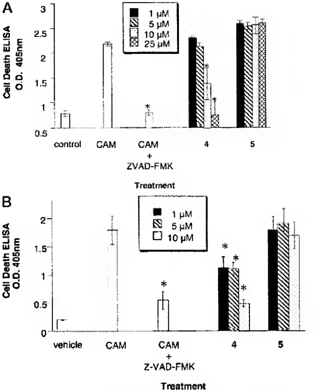


FIG. 4. **The effect of caspase 3/7-selective inhibitors on camptothecin-induced apoptosis of human chondrocytes.** Immortalized human C20/A4 chondrocytes (*A*) and primary bovine chondrocytes (*B*) were treated with camptothecin (CAM) (4 μg/ml) alone or in combination with varying concentrations of compound **4** or **5**, and cell death was measured and compared with the death of cells grown in serum containing medium alone (control). Lysates from approximately 5×10^4 cells were prepared, and cell death was measured by the cell death ELISA. Results are mean ± S.D. ($n = 3$) ($* = p < 0.02$, compared with CAM treatment alone).

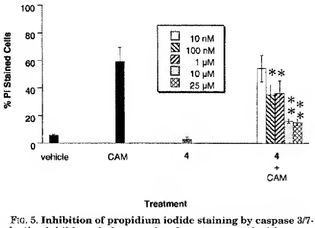


FIG. 5. **Inhibition of propidium iodide staining by caspase 3/7-selective inhibitors in human chondrocytes treated with camptothecin.** Chondrocytes (C20/A4) were treated overnight with compound **4** plus or minus CAM (4 μg/ml). Propidium iodide staining was measured, and compound **4** dose-dependently inhibited staining induced by overnight treatment with CAM. Results are mean ± S.D. (n = 3) ($* = p < 0.02$, compared with camptothecin treatment alone).

drocytes. Following the induction of apoptosis, type II collagen promoter activity markedly decreased, suggesting a shutdown of chondrocyte matrix production (Fig. 6, *A* and *B*). The non-selective peptide inhibitor Z-VAD-FMK was able to significantly inhibit the loss of type II collagen promoter activity observed after induction of apoptosis. Similarly, the caspase 3/7-selective inhibitor **4** blocked the decrease of type II collagen promoter activity (Fig. 6, *A* and *B*). No effect was shown by compound **5**, a significantly less active compound against the isolated enzymes. These data suggest that the caspase 3/7-selective inhibitors block apoptosis *in vitro* yet maintain the transcriptional activity of the chondrocyte-specific type II collagen promoter.

DISCUSSION

The isatin sulfonamides are the first reported examples of potent and selective inhibitors of caspases 3 and 7 and represent a novel strategy for the development of active agents which block apoptosis. In contrast to currently available agents, these compounds provide the opportunity to evaluate the inhibition of effector caspases in cell-based models of apoptosis. Previous inhibitor studies have suggested that binding in

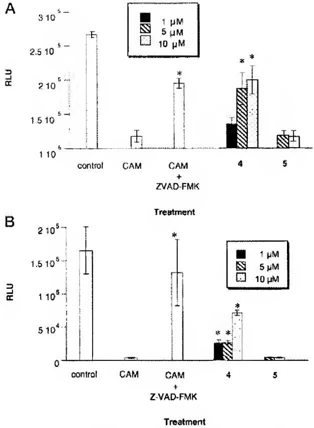


FIG. 6. **The maintenance of type II collagen promoter activity after apoptotic insult in the presence of the caspase inhibitors and Z-VAD-FMK.** The C20/A4 chondrocyte cells stably transfected (*A*) and primary bovine chondrocytes transiently transfected (*B*) with a construct in which the regulatory sequences (−577 to +3426) of the type II collagen (*COL2A1*) gene are driving luciferase reporter gene expression. Cells were grown in serum-containing medium as a control (control). Cells were treated with 4 μg/ml CAM or 4 μg/ml CAM plus varying concentrations of compound **4** or **5** for 24 h. Expression of the *COL2A1* gene was measured by luciferase activity measurements using a luminometer. Results are expressed as relative light units (*RLU*). Results are mean ± S.D. ($n = 3$) (* = $p < 0.02$, compared with CAM treatment alone).

the S_1, S_3, and S_4 subsites is critical for potent and selective inhibition of caspases. Binding to the S_1 subsite confers selectivity for caspases, while binding to the S_3 and S_4 subsites is generally believed to be critical for selectivity between caspases (13). The x-ray crystal structure of the complex between inhibitor 4 and caspase 3 shows minimal inhibitor interactions with these subsites. Instead, selectivity is obtained via extensive hydrophobic contacts between the pyrrolidine ring of the inhibitor and residues Tyr^{204}, Trp^{206}, and Phe^{256} of the S_2 hydrophobic pocket. Thus, the isatin sulfonamides are the first examples of potent caspase inhibitors that achieve their selectivity through interaction with the S_2 subsite.

Although our initial goal was to identify a selective inhibitor of caspase 3, the effort yielded inhibitors with dual selectivity for caspases 3 and 7. However, caspases 3 and 7 are frequently activated at similar time points during the apoptotic signal transduction cascade and may play non-redundant roles in the processing of downstream substrates. Since the isatin sulfonamides offer the ability to block both of these effector caspases, we believe these compounds may possess advantages over a caspase 3-selective inhibitor.

The selectivity of isatin sulfonamide 4 for caspases 3 and 7 permitted the demonstration that inhibition of these caspases results in the blocking of cell death in two apoptotic cell models, one of which has been shown to previously to be absolutely caspase 3-dependent (4, 5). Inhibition of mouse bone marrow neutrophil apoptosis with caspase 3/7-selective inhibitors is in agreement with the previous report from caspase $3^{ex3-/-}$ mutant mice, which have been shown to be resistant to apoptotic insult (4, 5). This result suggests that the mechanism by which these compounds exert their anti-apoptotic activity is by inhibition of caspase 3.

We next showed that chondrocyte apoptosis was also blocked by the caspase 3/7-selective inhibitors. The extracellular signals that stimulate chondrocyte apoptosis *in vivo* and the intracellular pathways triggered that ultimately result in DNA fragmentation and cell death are not well understood. Chondrocyte apoptosis can be triggered *in vitro* by multiple stimuli, which may reflect local physiological conditions during the development and/or progression of OA, *e.g.* enhanced local cytokine production (TNF-α) and reduced growth factor concentrations (low serum) (42). Increased cytokine levels and reduced growth factor environment have been reported in aged/diseased articular joints (42). Interleukin-1 and TNF-α are cytokines that have been proposed to play a role in both inflammatory and non-inflammatory joint diseases such as rheumatoid arthritis and OA and have both been reported to stimulate chondrocyte apoptosis (15, 43). In addition, we have also shown that multiple stimuli, including TNF-α and growth factor withdrawal, induce caspase 3 activity in human and bovine chondrocytes (50).

Chondrocytes are responsible for the production and maintenance of the extracellular matrix, which functions to allow optimal fluid joint articulation. Therefore, inhibition of cell death alone may not be sufficient for a beneficial effect on the articular cartilage. Ideally, an anti-apoptotic agent would be required to prevent cell death yet maintain the chondrocyte as a matrix producing cell. Inhibition of apoptosis with the caspase 3/7 inhibitors does just that. In our studies, prevention of apoptosis by inhibition of caspase 3/7 activity resulted in maintenance of the transcriptional activity of the *COL2A1* promoter. Maintenance of type II collagen promoter activity suggests that expression, of this major cartilage matrix protein, would be maintained in the presence of the inhibitors. Therefore, in a catabolic state an anti-apoptotic agent may allow the cells, which were destined to die, to survive and more importantly to continue to synthesize and deposit new matrix. A major issue in treating chronic degenerative diseases will be to prevent the unwanted death of cells while not allowing the proliferative diseases, such as cancer or rheumatoid arthritis-associated synovial hypertrophy (44, 45), to flourish. Therefore, understanding the role of the specific apoptotic proteases and their aberrant function in these highly proliferative cells is an important challenge. If cancer cell survival is indeed enhanced by non-selective caspase inhibition, then therapeutic approaches will need to be selective for the target cell, so that general cellular hypertrophy is not stimulated. For inhibition of apoptosis to be therapeutically beneficial, rescued, "non-dead" cells must function in a normal manner. A report by Davidson and Steller (46) described residual cell function in *Drosophila* retinal degeneration mutants, which have a condition similar to human retinitis pigmentosa. The cell survival protein p35 blocked apoptosis in the mutant photoreceptor cells, and the flies retained more visual function than mutant, untreated flies. This model suggests that late stage (mature) anti-apoptotic therapeutic strategies may be effective against chronic degenerative diseases.

Our data suggest that inhibiting chondrocyte cell death with caspase 3/7-selective inhibitors may provide a novel therapeu-

tic approach for the prevention and treatment of OA or other disease states characterized by excessive apoptosis that involves the caspase 3/7 pathway.

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